REVIEW

Recent Advances in Transgenic Technology

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Abstract

Techniques that allow modification of the mammalian genome have made a considerable contribution to many areas of biological science. Despite these achievements, challenges remain in two principal areas of transgenic technology, namely gene regulation and efficient transgenic livestock production. Obtaining reliable and sophisticated expression that rivals that of endogenous genes is frequently problematic. Transgenic science has played an important part in increasing understanding of the complex processes that underlie gene regulation, and this in turn has assisted in the design of transgene constructs expressed in a tightly regulated and faithful manner. The production of transgenic livestock is an inefficient process compared to that of laboratory models, and the lack of totipotential embryonic stem (ES) cell lines in farm animal species hampers the development of this area of work. This article highlights recent progress in efficient transgene expression systems, and the current efforts being made to find alternative means of generating transgenic livestock.

Index Entries: Transgenic; review; mouse; gene regulation.

1. Introduction

For over a decade, researchers have been able to manipulate directly the genome of mammalian species, allowing scientists in a diverse array of disciplines to advance their understanding of complex systems. The production of the first transgenic animals represented a successful alliance between the disciplines of developmental and molecular biology. During the 1960s and 1970s, embryologists had been studying the physiology of preimplantation mouse embryos and perfecting the culturing conditions for successful embryo development. Such work laid the foundation for both IVF/assisted reproduction programs and transgenic technology. The development of micromanipulation techniques together with studies on the capacity of preimplantation embryos to transcribe and translate exogenously introduced genetic material rapidly led to the production of the first transgenic mice in 1980 *(1)*. Within months four other groups had reported the birth of animals containing foreign gene sequences, and it became clear that introducing genetic material into the pronucleus of one-cell mouse embryos resulted in the integration of these sequences at an early stage of development and their retention beyond birth *(2–5)*.

The implications of such work were quickly grasped by the wider scientific community. The potency of this new area of science was demonstrated in 1982 when Palmiter et al. introduced growth hormone sequences under the control of the metallothionein promoter *(6)*. Some of the resultant transgenic lines showed markedly increased growth hormone levels in mice that grew to twice the size of nontransgenic littermates, indicating the degree to which physiological systems could be influenced by the expression of an exogenous gene. Such innovative studies encouraged the development of transgenic work in domestic animals. By 1985, the production of transgenic rabbits, sheep, and pigs

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had been achieved *(7)*, and the first transgenic cattle were reported in 1989 *(8)*. However, the overall efficiency of livestock production was considerably reduced compared to mice. This together with the high capital cost and the long generation interval of domestic animals has hampered research in this area. On the other hand, transgenic laboratory models have flourished, and the initial promise of this technology has been amply demonstrated.

2. Flexibility of the Transgenic Approach

Although the principal application of transgenic work is the investigation of gene function and gene regulation—usually within a specific tissue context—there are a number of other uses to which the technology can be put. This approach has been particularly fruitful when used to address complex areas, such as oncology or immunology. For example, overexpressing a putative oncogene within a particular tissue compartment can yield information regarding the biological function of the oncogene and the susceptibility of that tissue for transformation. Furthermore, such mice represent a model for examining oncogene cooperation and the other events that can contribute to this multistep process. Similarly, key experiments using rearranged T-cell receptors or immunoglobin genes have been highly informative with regard to the selection processes involved in moulding the immune repertoire.

The physiological role of particular cell lineages can be examined by expressing cytotoxic proteins that ablate a specific tissue compartment *(9)*. This has been achieved with a number of different tissue types and has been particularly useful in exploring cell–cell interactions and cell-lineage relationships.

An additional byproduct of transgenic production is the creation of novel mutants. Transgene integration can act as a mutagenic event, and around 5–15% of transgenic lines show phenotypic evidence of insertional mutagenesis when homozygous for the transgene *(10)*. In these circumstances, the presence of the transgene acts as a tag that can assist the genetic analysis of the mutation (for review, *see* ref. *11*).

Gene knockout experiments, using gene targeting protocols in embryonic stem (ES) cells, have proven to be a potent technique in understanding gene function, particularly in the areas of developmental biology and immunology. These studies have emphasized the extent of genetic redundancy within mammalian systems. Frequently, the deletion of a gene thought to have an important role results in mice with either no phenotype or only very subtle phenotypic changes. This appears to be owing to the ability of related genes with overlapping functions to compensate for the loss of the targeted gene. As the technology becomes more accessible, growth in this area of work is expanding rapidly, although attempts have been made to establish a data base of knockout mice *(12–14)*.

The introduction of reporter genes into ES cells can be used to randomly "trap" and mutate novel genes. Genes initially characterized by their temporal and spatial expression patterns together with an interesting mutant phenotype can be identified owing to the presence of the transgene insert. Vector design and the protocols used to identify endogenous genes vary. Constructs lacking a promoter express the reporter gene when they insert within an exon of a host gene, whereas constructs carrying a splice acceptor site generate a fusion product with the endogenous gene, thus allowing the mutated gene to be cloned *(15)*.

There are a number good reviews dealing with the impact of transgenic technology in different areas of biomedical research, including: immunology *(16–18)*, oncology *(19–24)*, neurological disease *(25)*, reproduction *(26,27)* and disease models *(28–30)*. In this article, I will concentrate on the recent advances that have improved the utility of transgenic technology in both laboratory models and livestock.

3. Methods of Production

3.1. Pronuclear Microinjection

The original and most straightforward method of producing transgenic animals is pronuclear microinjection. This technique involves injecting a solution of DNA into one of the two pronuclei of the fertilized one-cell embryo. Using this

method, multiple copies of the gene construct integrate, usually in long head-to-tail tandem arrays. Normally, a single integration event takes place, but occasionally a transgenic founder will carry two separate integrations, resulting in the establishment of two distinct transgenic lines from this animal. A small proportion (20–30%) of animals will produce significantly less transgenic offspring than expected from Mendelian principles. In these animals, integration into the host genome has occurred beyond the one-cell stage, resulting in mosaicism. In 1985, Brinster et al. rigorously investigated those factors that influenced the efficiency of pronuclear microinjection, and found that both DNA concentration and DNA form significantly affected the success of the procedure *(31)*. The genotype of the mouse employed was also important with F1 hybrids proving to be much more robust and overall a much more efficient resource than inbred lines.

The process of microinjection results in a proportion of embryos being lysed at the time of injection. Further, the long-term survival appears to be compromised as embryo loss continues through preimplantation and fetal development, presumably as a result of widespread genetic damage *(32)*. As a result, only a proportion (15–25%) of microinjected mouse embryos survive to term. The proportion of mice that ultimately prove to be transgenic also varies between groups and even between different experiments, but 10–40% is within the normal range *(10)*.

3.2. Retroviral Vectors

Retroviruses have a unique lifestyle: following infection, a double-stranded DNA copy of the RNA genome is produced by the virus-specific enzyme reverse transcriptase *(33)* and integrates into the genome of the host cell. The capacity of retroviruses to infect nonlytically a wide range of cells and integrate efficiently a single copy of their genetic material has made them an ideal vehicle for gene transfer experiments. In 1975, Jaenisch and colleagues showed that mouse preimplantation embryos were susceptible to infection with murine leukemia virus (MLV) and subsequently reported that proviral sequences could be retained within the germ line of mice infected at this stage *(34,35)*. Retroviral vectors can be prepared using packaging cell lines that express the viral structural proteins *in trans*. Introducing the gene of interest flanked by *cis*-acting retroviral sequences that package the RNA results in the production and shedding of virus vector (for review, *see* **ref. 36**). Retroviral vectors have been used to transfer foreign genes into the germ line of mice *(37,38)*. Infection of early cleavage stage embryos often produces a mosaic pattern of proviral integrations in the resulting offspring, but these segregate in subsequent generations. A major constraint with this approach is that there is an upper limit to size of the gene construct that can be incorporated into the retroviral particle. In addition, the necessity for designing an appropriate vector and the use of packaging cell lines makes it a more cumbersome approach, and this has limited its use in mice. However, in those species, such as chickens, where pronuclear microinjection is not as efficient, retroviral vectors have been widely used *(39,40)*. A pilot study conducted in the late 1980s showed that wild-type feline leukaemia virus (FeLV) was capable of infecting ovine embryos *(41)*. This technique has not been widely applied partly for the reasons discussed above and partly because of concerns over the safety of using retroviral vectors in domestic animals. However, more recently Kim et al. have used this approach in cattle, and showed that it was possible to introduce and express a retroviral vector containing the β-galactosidase gene by coculturing zona-free embryos over packaging cell lines *(42)*. This experiment exploited a replication defective MLV vector that carried the Gibbon leukaemia virus envelope.

3.3. ES Cells

The most powerful and elegant method for modifying the mouse genome is gene targeting in ES cells. With this approach, not only can exogenous genes be added, but endogenous genes can also be functionally deleted or specifically mutated. ES cell lines are totipotential cells derived from explanted blastocysts *(43,44)*. Under appropriate conditions, these cells can be grown indefinitely

and remain in an undifferentiated state. Introduction of ES cells into the blastocoele cavity of preimplantation embryos can result in adult chimeric animals *(45)*. Owing to the totipotential nature of these cells, they can contribute to the development of the various cell lineages, including the germ line. As a result, subsequent breeding can produce lines of mice that are genotypically derived from the ES cells.

DNA preferentially interacts with stretches of homologous DNA (homologous recombination). This property can be exploited to target specific endogenous genes with the introduced genetic material *(46–48)*. Despite this, the frequency of homologous integration is still 10^2- to 10^4-fold less than that of random integration. Although targeted gene interaction has been achieved by pronuclear injection, the low incidence of this event means that for practical purposes, some form of in vitro selection has to be applied. Selection protocols to isolate and clone ES cells that have integrated the introduced DNA and sustained successful targeting have been developed *(49–51)*.

4. Transgene Regulation and Expression

It is possible to direct transgene expression to particular tissues by fusing tissue-specific promoters to the gene of interest. Early reports include targeting gene expression to the pancreas using both the insulin gene *(52)* and an elastase-human growth hormone gene *(53)*. Tissue-specific expression was also recorded in the lens of the eye using the murine α-A-crystalline promoter *(54)* and muscle using an chimeric actin-globin gene *(55)*. Examples of temporal control of expression have also been reported with the α-fetoprotein gene being expressed in a manner analogous to the endogenous gene *(56)*. Human fetal globin transgenes introduced into mice were also regulated in a temporal manner *(57)*. Transgenic mice have therefore proven to be a useful model system for testing a wide variety of regulatory sequences and have helped unravel the complex hierarchy of the various elements involved in gene regulation.

Well-regulated transgene expression is the key to successful transgenic work, but all too often experiments are blighted by poor levels or the complete absence of expression, as well as le common problems, such as leaky expression i nontargeted tissues *(58)*. A feature common many transgenic experiments is the unpredictab nature of transgene expression with differe transgenic lines produced with the same constru frequently displaying different levels of expre sion. Further, expression levels do not correla with the number of transgene copies integrate *(59)*. Such copy-number-independent, integra tion-site-dependent expression patterns empha size the influence of surrounding chromatin on th transgene. This effect was elegantly demonstrate by Allen et al. *(60)*, who attached a relativel weak promoter to the *lacZ* reporter gene, resul ing in a series of transgenic lines with markedl diverse tissue and temporal expression patterns.

4.1. Use of Tertiary Regulatory Elements

It is now clear that position effects, which ar generally of a suppressive nature, are the result c the combined effects of chromatin configuratio at the site of integration, coupled with the absenc of "higher" *cis*-acting regulatory elements withi the transgene construct. As a result, the transgeni transcription unit is not sufficiently insulate from the effects of surrounding DNA. The impor tance of additional, if unidentified, regulatory ele ments was demonstrated by Brinster et al., wh showed that expression levels of genomic trans genes were generally superior to their cDN equivalents *(61)*.

Given the difficulties in achieving reliable an predictable expression, a report from Grosveld e al. describing high-level expression in ever transgenic line produced was received with mucl interest *(62)*. These workers used a β-globin gen construct containing extensive flanking sequences and found that the distinctive erythroid specifi expression pattern that resulted was owing to novel regulatory element buried within the flank ing sequence. Termed a locus control regio (LCR), this element operates some distance fron the coding regions and, in the case of the β-globi gene locus, is characterized by a cluster of fiv hypersensitivity (HS) sites. Since then a numbe of tissue-specific LCRs have been identified, includ

ing the CD2 *(63)*, α-hemoglobin *(64)*, lysozyme *(65)*, metallothionein *(66)*, tyrosinase *(67)*, and the major histocompatible complex loci (68). These elements are defined by their ability to confer high-level, position-independent, and transgene-copy-number-dependent expression of linked transgenes. It has been postulated that LCRs overcome the generally negative effects of position by establishing open chromatin domains *(68)*. Many of these elements remain poorly characterized, but some general points have emerged. LCRs are associated with a number of DNase HS sites that are essential, but not sufficient for locus control function. Developmental regulation of multigene loci depends on a complex interaction among the various HS sites found within the LCR *(69)*. It is now becoming clear that an LCR is not a single regulatory element, but rather represents a composite unit, which collectively is responsible for the properties that define an LCR. For example, mutational analysis of the LCR associated with the keratin 18 gene has shown that mutations that abolish copy-number-dependent expression do not necessarily affect site-independent expression, revealing that these two properties can be dissociated *(70)*. LCRs therefore contain elements that confer classical enhancer activity as well as elements that insulate the transgene from the effects of surrounding chromatin.

Distinct from LCRs, but with overlapping functions are elements known as matrix attachment regions (MARs) or scaffold attachment regions (SARs). It is thought that some MARs may represent the boundaries of chromosomal domains and, as such, act to insulate gene expression patterns between adjacent domains. These elements do not display classical enhancer activity, and their incorporation does not increase gene expression in transient transfection assays. Their inclusion within transgene constructs, however, has conferred position independence in stably transfected cell lines *(71)* and transgenic mice *(72)*. Zhoa et al. *(73)* have proposed that MARs may act as nucleation sites for histone proteins and that these proteins can be displaced by tissue-specific factors that initiate chromatin opening.

Additional control elements, termed facilitators, associated with human adenosine deaminase (ADA) gene regulation have also been found to have an important role in transgene insulation. The functional capacity of these elements appears to involve a very tight spatial relationship with the ADA enhancer. A series of experiments revealed that high-level, position-independent expression only occurred if these elements bilaterally flanked the enhancer domain, were in the correct orientation, and were located in specific positions relative to the enhancer *(74)*. The stereoscopic positioning of these facilitators suggests that they are involved in mediating structural changes to the DNA that permit enhancer activity.

4.2. Variegated Expression Patterns

Transgenic expression can be assayed using a variety of techniques, but these usually involve analysis of whole tissues. Examination of individual cells has revealed that suboptimal tissue expression can be due to a variegated pattern of expression within the target organ *(75)*. Removal of one of the three hypersensitivity sites contained within the CD2 LCR resulted in a proportion of transgenic lines displaying a variegated pattern of expression in the thymus *(76)*. This mutated LCR induced high-level expression, but only in a proportion of thymocytes. The decision to express or not appeared to be stochastic, occurred at an early stage of T-cell development, and was clonally stable. This expression profile was observed in transgenic lines carrying a centromeric insertion and may be related to cell-to-cell variation in chromatin condensation at specific chromosomal locations. Where transgene insertion occurred within euchromatic DNA, the mutant LCR was fully functional. These findings caused the authors to suggest that stochastic decisions affecting individual cells may be analogous to the events that allow a common precursor to differentiate along separate lineages. Variegated expression was also observed when a fully intact CD2 LCR was linked to a heterologous enhancer region, indicating that sequences within the "foreign" enhancer could modify the properties of the LCR *(77)*.

4.3. Yeast Artificial Chromosomes (YACs)

As a general principle, the greater the amount of DNA sequence associated with the coding gene—whether it be intronic or flanking DNA—the more likely transgene expression patterns will parallel the equivalent endogenous gene. However, distantly located regulatory elements, such as LCRs, have only been identified for a small number of genes, necessitating an alternative approach for the vast majority of experiments where faithful gene expression is crucial. YACs represent just such an alternative approach.

YACs have been introduced into the mouse germ line both by transfer to ES cells and by direct pronuclear microinjection where the efficiency of transgenic production is comparable to that achieved with standard transgene constructs. By employing YACs, transgene inserts in the 100–500 kb range have been successfully introduced, and the presence of YAC DNA within embryonic cells does not appear to interfere with development *(78–80)*. The major drawback with this approach is ensuring the integrity of the artificial chromosome. In general, the incidence of YAC deletions and rearrangements is greater when ES cells are used than when pronuclear microinjection is carried out. As a result, the overall efficiency appears to be greater with the latter technique *(79)*.

There are a number of advantages to using YACs as vehicles for gene transfer. This system can be used to investigate genes or gene complexes too large to be handled by standard transgenic protocols. Furthermore, by introducing the genomic transgene together with long stretches of flanking sequence, unidentified regulatory elements can be incorporated. With few exceptions, transgenes within the context of YAC DNA show spatial and temporal patterns of expression at levels comparable with the endogenous gene, indicating that this approach largely overcomes position effects *(79–81)*. A further advantage of using YACs is that yeast-based systems readily lend themselves to homologous recombination experiments and therefore facilitate the introduction of discrete mutations in the transgene. Bungert et al. employed this approach to investigate the role of individual hypersensitivity sites within the β-globi gene LCR *(82)*, whereas Peterson et al. hav examined mutations within the gene comple itself *(83)*. Pathogenic mutations have been intrc duced into the human amyloid precursor protei gene for Alzheimer modeling studies *(84)* an mutational studies on the human apolipoprotei B gene have identified the binding site for apolipc protein(a) using this technology *(85)*.

Molecular complementation of mouse mutant is also feasible using YAC transgenes *(86)*. Larg DNA fragments can be inserted into geneticall undefined mouse mutants in an attempt to revers the phenotypic consequences of the mutation an identify the affected gene. The ability to transfe and express very large fragments in the mous opens up hitherto inaccessible areas of investiga tion. Perhaps the best example are mice carryin large portions of the human immunoglobin gen locus in germ line configuration. In addition t immunoglobin rearrangement and selection, hyper mutation can occur in these transgenic mice. Sucl models may permit the efficient production o antigen-specific human antibodies *(87)*.

4.4. Gene Regulation Using Homologous Recombination

Although YACs represent one way to embec transgenes within extensive *cis*-acting sequence: a different approach is to use ES cells to targe transgenes to specific loci *(88,89)*. In this way the transgene can be "hooked up" to endogenou regulatory regions, thus paralleling the expressior pattern of genes situated at that chromosoma position. Such an approach has been used to inves tigate the redundancy of related genes that have temporally different patterns of expression *(90)*.

4.5. Inducible Gene Expression

The ability to induce or repress the transgene would be highly advantageous to many experiments. A definitive start point to transgene expression would permit a more stringent investigation of the phenotypic consequences of that expression. This would be particularly helpful in the study of complex lineages. Often tissue-specific promoters and enhancers are not sufficiently pre-

cise to target gene expression to a single developmental stage. For example, *de novo* induction of transgene expression within the T-cell compartment would allow the phenotypic consequences of expression in the different T-cell populations to be examined in isolation, rather that observing the phenotypic effects of sustained expression throughout development. External regulation would also prove useful in understanding complex genetic events, such as those involved in tumorigenesis. For example, the ability to repress transgenes involved in tumor initiation at later stages of the neoplastic disease process would help to identify which tumorigenic events are required for tumor maintenance *(91)*.

Inducible transgenic systems have been employed for some time with regulatory elements of the metallothionein gene being used in early transgenic experiments *(59)*. Unfortunately, there are a number of disadvantages associated with the majority of inducible elements, including pleiotropic or toxic effects of the inducing agent as well as high basal activity of the promoter. Some inducible promoters appear to work well with a low basal activity and substantial upregulation following induction, but their action is restricted to specific tissues (for review, *see* **ref. *92***).

Although still to be tested extensively with a variety of different constructs, a promising system has been described recently. Gossen and Bujard have developed a tetracycline responsive binary system, arguing that the inducers of conditional prokaryotic regulatory elements are less likely to have wider side effects in eukaryotic cells *(93)*. Two versions of the system have been described. The original approach involves fusing the *tet* repressor with the transactivating domain of the viral protein (VP16) of herpes simplex virus. The resultant transactivating protein is capable of binding to and activating a minimal promoter linked to *tet* operator sequences. Transactivation is blocked when tetracycline is present, and therefore the introduction of tetracycline acts as transcriptional repressor *(94)*. The alternative system utilizes a mutant *tet* repressor that has the opposite effect of the wild-type repressor in that it binds to the *tet* operator in the presence of tetracycline and, when converted, to an activator is capable of inducing expression when tetracycline is introduced (95). Using tetracycline to induce rather than repress expression is likely to be a more convenient approach in transgenic animals.

The *cre-lox* system permits activation of a quiescent transgene as well as conditional and cell-specific gene deletion. The Cre enzyme is a bacteriophage (P1) recombinase that can excise DNA flanked by specific *lox* sequences. These *lox* sites are comprised of two 13-bp repeats separated by an 8-bp spacer region. Transgenes can be designed in such a way that the gene can be activated by Cre-mediated removal of an internal DNA sequence *(96)*. This can be achieved by crossing transgenic lines carrying the dormant transgene with lines expressing the *cre* recombinase in specific tissues. An obvious advantage would be the establishment and maintenance of transgenic lines that would otherwise be unsustainable because of the deleterious effects of the transgene.

Investigating the phenotypic effects of gene deletion in specific tissues is frequently frustrated, because the absence of the gene results in embryonic or fetal death. The *cre-lox* system can also be used to delete genes by coexpressing the *cre* recombinase in specific tissues. Gene targeting of ES cells would thus be aimed at flanking the gene of interest with *lox* sequences rather than abolishing its function. This approach has been elegantly demonstrated by Kühn et al., who placed the *cre* recombinase under the control of the interferon-inducible promoter Mx *(97)*. Subsequent intercrossing with transgenic mice carrying an endogenous gene flanked by *lox* sequences results in efficient deletion of the targeted gene in specific tissues.

4.6. Dicistronic mRNA

There are a number of circumstances where it is desirable to coexpress two separate genes, usually the bioactive transgene and a reporter or selectable marker. Phenotypic analysis of tissues perturbed by transgene expression can be facilitated if those cells also express an easily detected histochemical marker. The standard protocol for expressing two separate gene products in transgenic mice is to coinject them as separate trans-

gene constructs. This usually results in the constructs integrating at the same locus. Alternatively, a single construct containing two discrete expression cassettes can be employed with transcription being driven by internal promoters or alternative splicing arrangements. However, obtaining reasonable levels of expression for both genes can be problematic. An alternative approach is to use internal ribosome entry sites (IRES) to generate dicistronic mRNA. IRES have been functionally defined in both viral and cellular systems, and provide an alternative translation initiation site to the 5'-cap structure *(98)*. Experiments involving retroviral vector infection of cell lines have shown the efficacy of expressing two gene products from a single transcriptional unit using IRES dicistronic constructs *(99)*. This system could also be advantageous when embedding transgene inserts within endogenous genes, either when using gene targeting in ES cells or when hooking up exogenous expression cassettes to genomic genes in YACs.

5. Progress in Transgenic Livestock Production

Although the first transgenic livestock were produced over a decade ago, progress in the intervening period has been slow. By comparison with mice, the efficiency of transgenic production in livestock remains relatively low with only around 1% of injected eggs resulting in transgenic offspring *(100)*. The reported figures with respect to cattle have been even poorer *(101)*. Only 15% of microinjected zygotes develop to the morula/blastocyst stage and only 18% of these survive to term. Further the integration rate in cattle experiments has also been low at around 3%. As a result, more than 1000 zygotes have to be injected for each transgenic calf produced—this in the species that is both the most expensive to use and has the longest generation interval. Costs can, to some extent, be reduced by the aspiration and in vitro maturation of oocytes derived from abattoir material, although subsequent development is poorer than that observed with in vivo derived fertilized ova. Allowing large numbers of microinjected embryos to develop in ligated rabbit or sheep uterine tubes before transfer significa reduces the number of recipients required. H ever, attempts to identify transgenic embr before transfer by using PCR have been probl atic, mainly because of the large number of f positives arising from the presence of unintegr DNA *(101)*. There is, therefore, a great need an alternative method of producing genetic altered livestock.

5.1. Utilization of Totipotential Cells for Transgenic Livestock Production

The development of farm animal ES cells can be manipulated in vitro, efficiently colo the germ line, and retain their totipotential st over a prolonged period is the focused objec of much research in the area. Numerous adv tages would accrue from this approach, includ a significantly increased efficiency in transge production, characterization of inserts bef reconstitution of host embryos and transfer, p venting the inadvertent mutation of an endogen gene, the ability to delete or modify endogen genes, and the capacity to target a genomic site would be permissive for expression.

ES-like cell lines have been developed fr cattle, sheep, and pig embryos. Cells derived fr the inner cell mass of bovine blastocysts can cultured for a limited period in suspension cult while remaining totipotential *(102,103)*. Ho ever, culture methods capable of supporting th ES cells for a prolonged period of time were able to sustain their totipotential properties *(1* Exciting progress has, however, been made w porcine ES cells. Wheeler has reported the d vation and long-term culture of ES cells from blastocysts *(104)*. These cells, which were kar typically stable, were capable of cooperating development. The efficiency of this proced was remarkably good, very few recipient blas cysts were damaged by manipulation, the e ciency of embryo transfer was comparable w that of nonmanipulated embryos, and the maj ity of the resultant offspring (72%) showed e dence of chimerism.

It may be possible to increase further the e ciency of producing genetically manipula

livestock by bypassing the chimeric generation. Ordinarily chimeric animals, resulting from the introduction of ES cells into host blastocysts, are bred to determine if the ES genotype has contributed to the germ line. In domestic animals, this would represent a further delay before "pure" ES cell-derived stock could be established. However, it may be possible to substitute the genetic material of the recipient embryo with that of the ES cells, thus avoiding the necessity for the intermediate chimeric animal. Sims and First have shown that cells cultured from the inner cell mass of bovine blastocysts can be used as nuclear donors *(102)* and that a proportion of these reconstituted embryos can develop to term *(103)*. DNA analysis of the calves revealed that the genotype was that of the cell line and not the host oocyte. However, the inner cell mass had only been cultured for a relatively short period of time. It remains to be seen whether this system will be sufficiently robust to produce long-lived cell lines that can be genetically manipulated in vitro.

Recently, Campbell et al. have described a modified approach *(105)*. This group has derived a novel epithelial-like cell line from the embryonic disks of d 9 sheep embryos (named totipotential for nuclear transfer or TNT). Initial experiments showed that nuclei from this line remained totipotential for up to three passages, but nuclei from passages 6–11 appeared to have lost the capacity to support embryonic development. However, cell nuclei from later passages were capable of supporting the development of enucleated oocytes following the induction of quiescence, although the reasons for this are unclear at present. The authors have suggested that chromatin from cells that have entered a state of quiescence may be more readily deprogrammed by oocyte cytoplasm. Alternatively, since quiescent cells are diploid, it may be that this state is more compatible with the host oocyte. Whatever the reason, this approach appears to be important in rescuing the totipotential properties of these cells and represents the first evidence that cells from an established cell line can produce live offspring following nuclear transfer.

6. Current and Future Applications of Livestock Transgenesis

As the technology involved in producing transgenic livestock becomes more accessible, a number of applications can be considered, such as the development of novel disease models (for review, *see* **ref.** *106*) and the creation of livestock lines resistant to specific animal diseases (for review, *see* **ref.** *107*). Two areas that have already made significant progress are pharmaceutical farming and xenotransplantation. Although obtaining high levels of biological active human proteins in the milk has not always proven to be easy (largely because of disappointing levels of expression), some groups have been successful (for reviews, *see* **refs.** *108,109*). Indeed, sheep expressing human α1-antitrypsin in the mammary gland have already reached the commercial stage.

The production of transgenic pigs has led the way in the use of genetically modified animals for xenotransplantation *(110,111)*. By expressing human complement regulatory proteins within transgenic pigs, it has been possible to reduce vascular damage associated with hyperacute rejection and significantly prolong the life-span of donor organs following transfer to primates. Although the complex processes involved in the rejection of discordant xenografts are not fully understood (for reviews, *see* **refs.** *112,113*), this work represents a major step forward in the use of animal organs for human transplantation.

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